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PRINCIPAL INVESTIGATOR: Irene E. Kochevar, Ph.D.

CONTRACTING ORGANIZATION:

Massachusetts General Hospital
Boston MA 02114-2621

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14. ABSTRACT

Purpose: The overall goal of this research is to preserve vision of patients recovering from severe facial burns by providing an improved method to reduce development of corneal defects, inflammation, infection and opacification. **Scope:** To further improve and understand the properties of the degradation-resistant crosslinked amniotic membranes for treating cornea of burn patients that were produced in Years 1 and 2. **Major findings**: Identified membrane stiffness as a critical parameter for a successful cornea-protective membrane and quantitatively assessed this parameter. Monolayer crosslinked amnions showed low stiffness and excellent enzyme degradation resistance. Tri-layer membranes showed excellent enzyme degradation resistance but their high stiffness made eliminated them from further consideration. Showed that two of the three crosslinking methods to produce stable membranes also decreased the levels of a soluble pro-healing factor. Constructed an amnion-encapsulated hydrogel lens with an improved enzyme resistance to be used to maintain hydration on the cornea surface of patients with peri-orbital scarring that prevents blinking.

15. SUBJECT TERMS
ectropion, cornea, burn scars, amniotic membrane, protein crosslinks, photomedicine, photochemistry, proteinases

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INTRODUCTION

The overall goal of this research is to improve the visual outcomes as well as the quality of life for burn patients during the acute and convalescent phases of their rehabilitation. Scarring from second and third degree facial burns, and from subsequent skin grafts, causes the tissues involved to contract and, if significant enough, the patient is left unable to blink or close their eyes. This results in desiccation of the ocular surface, breakdown of the cornea's defense mechanisms and subsequent events that may lead to cornea opacification and the need for cornea transplant. Currently these patients receive frequent application of topical lubricants and anti-inflammatory medications, an imperfect solution that requires very frequent administration of drops which keeps the wounded warrior at the bedside and can slow down other rehabilitation services that are remote to the inpatient ward. Amniotic membrane (AM) transplantation to the ocular surface can assist in the maintenance of the ocular surface of these patients. However, commercially available membrane is not only very expensive, but enzymes on the inflamed ocular surface degrade and destroy the AM very rapidly; this occurs in one day compared to two weeks in non-burn patients. In this project, our major goal is to stabilize the amniotic membrane by crosslinking it's constitutive proteins before applying it to the patient's eye. We will determine the crosslinking method that most effectively decreases the rate of enzymatic degradation of AM while preserving the beneficial anti-inflammatory and pro-healing factors in the membrane. In addition, we will evaluate photobonding as a sutureless, glueless alternative to sutures for attaching AM to cornea. We will also test an approach that combines AM with a hydrogel material to increase the ability of the amnion to hydrate the cornea. These studies will employ a rabbit model of eye inflammation.

BODY

This Grant Agreement is a joint proposal with COL Anthony J. Johnson, MD, PI on Grant Agreement W81XWH-09-2-0069. The Statement of Work includes tasks to be carried at the Massachusetts General Hospital (by Professor Kochevar's group) and the US Army Institute for Surgical Research (USISR) (by Dr. Johnson's group). This report covers results from Dr. Kochevar's lab and Dr. Johnson is submitting an separate Year 3 annual report. In this project period, Drs. Kochevar and Johnson discussed results and progress both in person (at the ATACCC meeting, August 2011 and at the annual meeting of the Association for Vision Research and Ophthalmology, April 2012) and during phone calls.

Objectives of the PRMRP were to enhance the translation of results from bench research to military clinical problems and, in turn, to facilitate learning fundamental information from medical applications. In accord with these objectives, we have made further progress in modifying amniotic membrane for protecting burn patients' corneas and the results obtained have lead to basic information regarding the processes involved in crosslinking proteins in tissue.

In Year 3, the studies on several sub-aims have been completed. Briefly, our results indicate that of the two approaches to producing a protective membrane from amnion, namely, crosslinked monolayer and crosslinking multilayer, only the monolayer material has the appropriate properties. We identified and quantified the stiffness of the crosslinked material as an additional critical property for a practical and useful protective membrane. In addition, the results demonstrated that a pro-healing factor in amnion, epidermal growth factor (EGF), is lost after treatment with two of the crosslinking agents (carbodiimide and genipin) and might be partially preserved after Rose Bengal photosensitization; this is being examined further in a no-cost extension period. Also, we demonstrated that carbodiimide crosslinking can be used to construct the encapsulated hydrogel contact lens for maintaining cornea hydration.

Specific aim 1.b. Determine whether a multilayer composite of amnion retards the rate of proteolytic degradation in vitro.

Our studies in year 2 had indicated that carbodiimide treatment of tri-layer amniotic membranes increased their resistance to enzymatic degradation, but the material appeared to be too stiff to conform to the curvature of the cornea. Since intimate contact is needed to bond the modified amnion to the cornea, a stiff highly crosslinked tri-layer membrane could not be used to protect the cornea. Thus, our research question became: Can a tri-layer crosslinked amnion membrane be produced that resists enzymatic degradation while remaining sufficiently flexible to ensure good contact with the cornea surface?

To address this question we quantitatively evaluated the stiffness of the crosslinked amnion and compared it to the percent enzymatic degradation of the same membrane.

Amniotic membrane
Amniotic membrane was prepared at the Massachusetts General Hospital as described in the Year 1 annual report and stored at -80°C until use.

Crosslinking with carbodiimide
The results obtained in Year 1 were used to select carbodiimide concentrations to crosslink amnion monolayers by a 1 h treatment followed by rinsing to remove remaining reagents and side products and drying. Carbodiimide concentrations were 0, 5, 9.4 or 10 mM in MES buffer. To produce amnion tri-layers, 3 circular discs of amnion (13 mm diameter) were placed between fine mesh circular brass screens with only the circumference secured. This arrangement allowed the reagents to diffuse into the membranes through the screens while keeping the layers in tight contact. The AM layers were stacked with each stromal surface in contact with the basement membrane surface of the next AM layer.

Enzymatic degradation
Bacterial collagenase was used to degrade proteins in amniotic membrane. Quantitative analysis of the small peptides released by degradation was accomplished using the fluorescence assay for free amino groups that was developed and described in our Year 1 annual report. The results are presented as the percent enzymatic degradation compared to untreated (non-crosslinked) control samples.

Stiffness measurements
The stiffness of a material (k) can be related to the elastic modulus (Young's modulus), measured as unconstrained uniaxial tension by: $k = (A \bullet E) / L$, where A is the cross-sectional area, E is the elastic modulus and L is the length of the element. We measured the elastic modulus of untreated and treated amnion with uniaxial tensiometry, then calculated the stiffness. This stiffness is not the truly the same as the ability of the amnion to bend to the contour of the cornea. However, the more realistic type of stiffness is not readily measureable, and it is not unreasonable to consider the stiffness we measured to correlate with the ability of the amnion to bend and conform to the shape of the cornea.

Uniaxial tensionmetry measurements were made on 5-mm-wide and 15-mm long strips of amnion (crosslink-treated or untreated). Each strip was mounted in the jaws of a Micro EP Miniature testing machine (Admet, Norwood MA) with a 10 N load cell. The initial distance between the jaws was 5 mm. A small drop of buffer was placed on each sample before beginning the measurement to ensure uniform hydration. Amnion strips were initially subjected to uniaxial tension (3 load cycles up to 0.01 N) before loading to failure. The stretch rate was set

at 1 mm/min. The axial tension load generated during the test was recorded (in Newtons, N) and the distance between the jaws (mm) was recorded using an MTESTQuattro controller (Admet, Norwood MA).

The stress was calculated by dividing the axial tension by the cross sectional area (thickness x 5 mm width) of each amnion sample. Strain was calculated by dividing the specimen elongation distance by its initial length. The elastic modulus was then calculated from the linear slope region of the stress-strain curve and used to calculate the stiffness (k) from the formula given above.

Amnion thickness measurement (collaboration with Conor Evans PhD and Yookyung Jung PhD) The thickness of each sample must be known in order to calculate the elastic modulus from stress-strain measurements as described above. Amnion varies in thickness between location on the membrane, between individuals and between methods for preparation. Typically, the untreated samples were between 30 and 60 μm thick. Amnion thickness was measured using optical coherence tomography (OCT) using a home-built ultrahigh resolution spectral domain system coupled to a custom inverted microscopy system. This OCT system utilized a 120 nm bandwidth superluminensent diode source centered at 855 nm to provide an axial resolution of approximately 3 μm. A pair of galvanometric mirrors coupled to a Ziess microscope using a .15NA Zeiss 5X objective was used to scan across the amion samples to collect xyz volumes. These volumes were then processed using a custom Matlab script that extracted amnion thickness measurements across entire 1.5 x 1.5 mm amnion samples.

Relationship between enzymatic degradation and amnion stiffness
Monolayer membranes-- Degradation of the membranes with collagenase was carried out in triplicate and the results are shown in Table 1, column 3 and Figure 1. Consistent with our previous results, increasing the concentration of carbodiimide decreased the extent of enzymatic degradation. Almost complete protection (95%) was produced by treatment with 10 mM carbodiimide.

Table 1 Carbodiimide crosslinking of amnion monolayers: Effect on enzymatic degradation and membrane stiffness.

Treatment	n	% enzymatic degradation ± SD	Thickness, μm ± SD	Stiffness, N/mm ± SD
Control, MES buffer	11	100 ± 5	42.2 ± 11.5	0.342 ± 0.156
5 mM carbodiimide	6	71 ± 4 *	26.8 ± 3.2 *	0.884 ± 0.284*
9.4 mM carbodiimide	13	18 ± 3 *	25.2 ± 3.7 *	0.821 ± 0.306*
10 mM carbodiimide	13	5 ± 3 *	20.2 ± 7.7 *	0.856 ± 0.487*

* indicates $p < 0.01$ compared to control.

The thickness of hydrated amnion monolayers, carbodiimide-treated or untreated, was measured (Table 1, column 4) and used to calculate the elastic modulus. The mean amnion thickness of the control amnion was 42.2 μm. All of the carbodiimide-treatments decreased the amnion thickness compared to the control group ($p < 0.01$) by 48-60%. There was no significant differences between the thicknesses of the carbodiimide-treated amnions although there is a trend to decreased thickness with higher carbodiimide concentrations. The decrease in

membrane thickness can be attributed to intra-fiber protein-protein crosslinks that decrease the distance between collagen molecules. Inter-fiber crosslinkis are not likely to form because the distance between collagen fibers in the amnion is too great to be bridged by the carbodiimide crosslinking which directly connects amino acids. Interestingly, even the lowest carbodiimide concentration decreased the amnion thickness substantially, indicating that the even a low amount of crosslinking can decrease the distance between collagen molecules. Further crosslinking within the fibers decreases the enzymatic degradation but did not produce significant further decrease in amnion thickness.

Treatment with carbodiimide substantially increased the stiffness of amnion monolayers. As shown in Table 1, column 5 and Figure 1, all three carbodiimide treatments increased the mean value for stiffness from 0.342 N/mm (untreated control) by ~40%**.** The stiffness values did not differ significantly between carbodiimide-treatments, but they all differed significantly from the control group ($p < 0.01$). Our previous studies (Year 2 report) indicated that amnion treated with similar concentrations of carbodiimide effectively bonded to cornea indicating that amnion with stiffness of ~0.8 N/mm are flexible enough to serve as protective membranes for corneas of ectropion patients.

Enzymatic degradation and stiffness show different patterns of response to treatment with increasing carbobiimide concentration; this is most clearly seen in Figure 1. The stiffness reaches a plateau starting at the lowest carbodiimide concentration (5 mM). Thus, the higher concentrations of carbodiimide that lead to nearly complete protection of the amnion from degradation do not further increase the amnion stiffness. This is good news since it means that we can vary the degree of protection against enzymatic degradation without being concerned that the monolayer membrane will be too stiff to bond to the cornea.

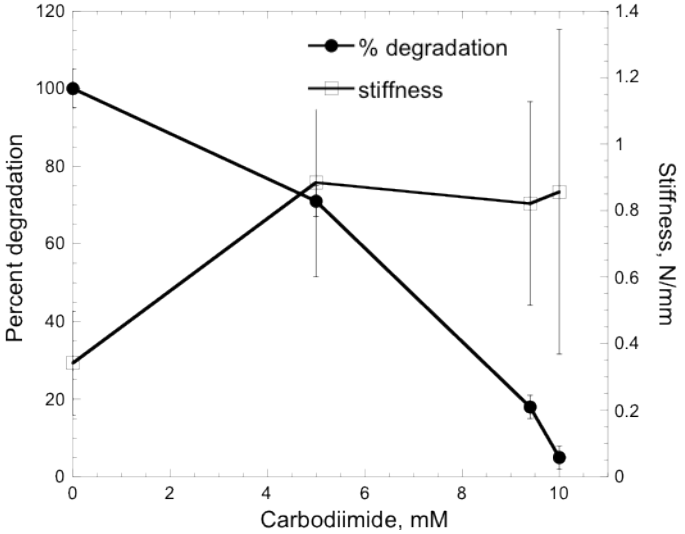


Figure 1. Effect of crosslinking monolayer amnion with carbodiimide on degradation by bacterial collagenase and on membrane stiffness. Amnion samples were incubated for 1 h with varying concentrations of carbodiimide, then degraded with bacterial collagenase or used for uniaxial tensiometry measurement of the elastic modulus, which was used to calculate stiffness.
* indicates $p < 0.01$ compared to control (no carbodiimide).

We then tested the tri-layer amnion that had been treated with 8 mM carbodiimide for 1 h (n = 8), conditions which had been shown in our previously reported study to decrease enzymatic degradation to 3% of the control value. Tensiometry was carried out as described above and the thickness of the tri-layer membrane measured with a digital caliper. The stiffness was 1.44 ± 0.51 N/mm. This value is substantially higher than the stiffness of the monolayer amnion (Table 1). Since the tri-layer membrane was too stiff to conform to the shape of the cornea and prevented the membrane from being photobonded to the cornea (Year 2 result), this result suggests that our stiffness measurements correlate with the ability of crosslinked to be bonded

to the cornea. This correlation will be further evaluated in the no-cost extension period by directly comparing the stiffness values to the strength of photobonding of the crosslinked membranes to cornea as measured in Year 2.

An important additional outcome of this study will be that we will have a physical measurement to quantitatively assess the stiffness of membranes produced after various crosslinking treatments. This will allow us to assess whether a modified membrane is sufficiently flexible, i.e., non-stiff, to conform to the shape of the cornea, thus reducing the amount of ex vivo or in vivo testing on rabbit eyes.

Summary/ Conclusions
The stiffness of crosslinked membranes was measured as a critical property for success of cornea protective membrane. Monolayer crosslinked amnion remained sufficiently flexible to allow production of highly stable crosslinked membranes that can be used for cornea protection. For tri-layer crosslinked amnion, high stiffness was associated with membranes that showed low enzymatic degradation, indicating that the tri-layer construct is not suitable for this application.

Specific aim 1.e.i. Identify the protein crosslinking method that causes the least reduction in anti-inflammatory and healing factors in amnion.

The ideal modified amniotic membrane for protection of ectropion eyes would contain the protein factor believed to contribute to the pro-healing and anti-inflammatory properties of amnion. In Year 2, we assessed the effect of amnion crosslinking techniques on the levels of TGF-β1, a proposed pro-healing factor in amnion. Our results showed that the crosslinking treatment conditions that produce degradation-resistant amnion also caused almost complete loss of TGF-β1. Because TGF-β1 is stored in tissue linked to LTBP (latent TGF-β binding protein) and closely associated with extracellular matrix proteins, we postulated that it became crosslinked to LTBP and/or the extracellular matrix proteins. Thus, TGF-β1 in crosslinked amnion would not be available for the healing process and could not be measured in our ELISA assay.

We had proposed to test the effect of amnion crosslinking treatments on a pro-healing protein that, in contrast to TGF-β1, is not stored in close association with extracellular matrix proteins in tissue. Epidermal growth factor (EGF) was selected as a representative of this group of proteins. This study was carried out using carbodiimide and Rose Bengal photosensitization as the crosslinking treatments. As will be shown below, these treatments were not satisfactory and, consequently, we also evaluated another protein crosslinking agent, genipin, that links proteins together by a mechanism that differs from carbodiimide and RB photosensitization.

Effect on EGF level of crosslinking amnion monolayers with carbodiimide or Rose Bengal photosensitization
The methodology for extracting soluble proteins from amnion developed in Year 2 was followed. Briefly, weighed amnion samples (treated or untreated) were homogenized at liquid nitrogen temperature, PBS and protease inhibitor were added and the mixture centrifuged. The supernatant was concentrated and the protein level measured. EGF was measured by ELISA using a commercial kit (R&D Systems, # DY236) according to the manufacturers directions.

Weighed samples of amnion (~25 mg) were treated with carbodiimide (0, 4, 6, 8 mM) for 1 h in MES buffer or treated with 0.1% Rose Bengal in PBS for 5 min, then exposed to 0, 5 or 50

J/cm^2 532 nm light from a cw KTP laser, as reported previously. A weighed portion (~1 mg) was used to measure the collagenase degradation as described above and the remainder was used for EGF measurement.

Crosslinking by treating with increasing concentrations of carbodiimide decreased both the percent enzymatic degradation and the EGF content of the amnion (Table 2, column 3 and Figure 2). The EGF content decreased more rapidly than the percent degradation as seen clearly in Figure 2. For example, treatment with 6 mM carbodiimide reduced EGF to ~ 12% of the MES buffer control whereas the enzymatic degradation was only reduced to ~30%. Samples showing ~90% protection against enzymatic degradation (treatment with 8 mM carbodiimide) retained only ~5% of the EGF originally present (an undesired outcome). These results are similar to those reported in Year 2 for the effect of carbodiimide crosslinking on TGF-β1 in amnion. Thus, carbodiimide crosslinking has the advantages of greatly decreasing the enzymatic degradation of amnion and producing a membrane that is not too stiff to conform to the contour of the cornea surface but appears to reduce the pro-healing activity of the amnion.

Crosslinking amnion by Rose Bengal photosensitization treatment also appeared to decrease the EGF level (Table 2) although this result is difficult to interpret firmly. In this experiment, Rose Bengal (RB) in PBS (no irradiation) decreased the apparent level of EGF by a factor of ~2.5 compared to a PBS control suggesting that RB interfered with the ELISA measurements. Irradiation with green light appeared to decrease further the EGF level, although some EGF remained, suggesting that RB photosensitization may be a useful crosslinking method. The enzymatic degradation measurements also seemed to be confounded by the presence of RB. We will remove the RB remaining after crosslinking treatments (or control PBS) from the samples by dialysis before ELISA and enzymatic degradation measurements to remove this interference. These studies will be carried out the no-cost extension period that has been approved for this award.

Table 2 Effect of carbodiimide and Rose Bengal photosensitized crosslinking of amnion monolayers on EGF content and degradation by collagenase.

n = 4 for enzymatic degradation; n = 3 for EGF ELISA
* = $p < 0.01$ compared to MES buffer control
† = $p < 0.01$ compared to RB only control

Treatment	% enzymatic degradation ± SD	EGF, pg/mg amnion ± SD
Carbodiimide treatment		
Control, PBS	100 ± 7	1.82 ± 0.04
Control, MES buffer	77.5 ± 19.8	1.83 ± 0.06
4 mM	44.3 ± 17.8 *	0.419 ± 0.089 *
6 mM	30.8 ± 19.7 *	0.225 ± 0.104 *
8 mM	10.9 ± 2.2 *	0.117 ± 0.175 *
Rose Bengal + 532 nm treatment		
RB only	50.9 ± 7.4	0.708 ± 0.365
RB + 5 J/cm^2	34.4 ± 23.0	0.317 ± 0.335 †
RB + 50 J/cm^2	57.7 ± 16.8	0.180 ± 0.094 †

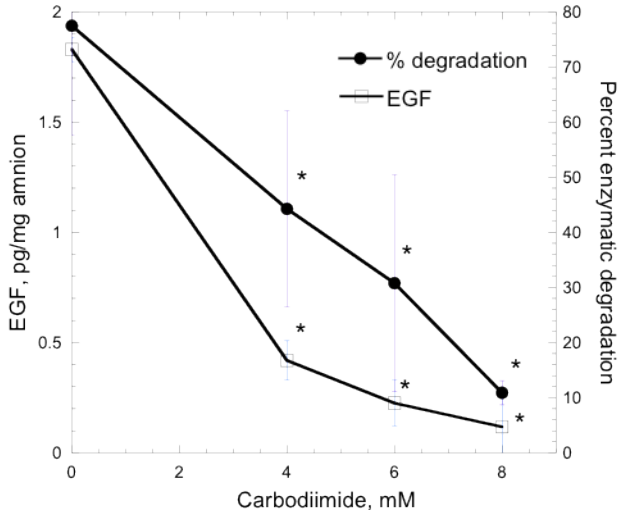


Figure 2 Effect of crosslinking monolayer amnion with carbodiimide on EGF levels and degradation by collagenase. Amnion was incubated for 1 h with varying concentrations of carbodiimide, then degraded with collagenase or EGF measured by ELISA.
* indicates $p < 0.01$ compared to MES buffer control.

Crosslinking proteins in amnion with genipin

Since carbodiimide (and possibly RB photosensitization) decreased pro-healing factors in amnion we tested whether an alternative protein crosslinking agent, genipin, might not alter the EGF level. First, the effect of genipin crosslinking on degradation of amnion by bacterial collagenase was evaluated. As shown in Table 3, column 3, increasing the time of incubation of amnion with 0.1% genipin (in PBS) decreased its susceptibility to enzymatic degradation, reaching 14% after 20 h incubation, a level that would be acceptable for a material to protect cornea of ectropion eyes.

Table 3 Genipin crosslinking of amnion monolayers: Comparison of effect on enzymatic degradation and membrane stiffness.

Treatment	n	% enzymatic degradation ± SD	Thickness, μm ± SD	Stiffness ± SD
Control, buffer only	11	100 ± 4	42.2 ± 11.5	0.342 ± 0.158
Genepin, 7 h	11	75 ± 8 *	32.9 ± 6.9 *	0.921 ± 0.331 *
Genepin, 18 h	11	27 ± 2 *	26.3 ± 3.6 *	0.778 ± 0.391 *
Genepin, 20 h	5	14 ± 3 *	25.2 ± 2.2 *	0.632 ± 0.156 *

* indicates $p < 0.01$ compared to control.

Initial, qualitative observations suggested that the genipin-crosslinked membranes appeared to be less stiff than those produced by carbodiimide treatment. Consequently, we measured the stiffness of the genipin-treated membranes using tensiometry and OCT as described above. However, the stiffness produced by genipin crosslinking was similar to the results obtained with carbodiimide. The range of values for stiffness (0.63 – 0.92 N/mm) for genipin crosslinking was similar to that obtained for carbodiimide (~0.84 N/mm). The relationship between degradation by collagenase and stiffness (Figure 3) was also similar to that found for carbodiimide crosslinking (Figure 1). Increasing the treatment time with genipin increased the protection against enzymatic degradation, i.e., less degradation, but did not increase the stiffness. These results indicate that highly stable crosslinked amnion monolayers can be produced with genipin

crosslinking that are not too stiff to be photobonded to cornea and thus may be an alternative to carbodiimide for producing membranes for cornea protection in ectropion eyes.

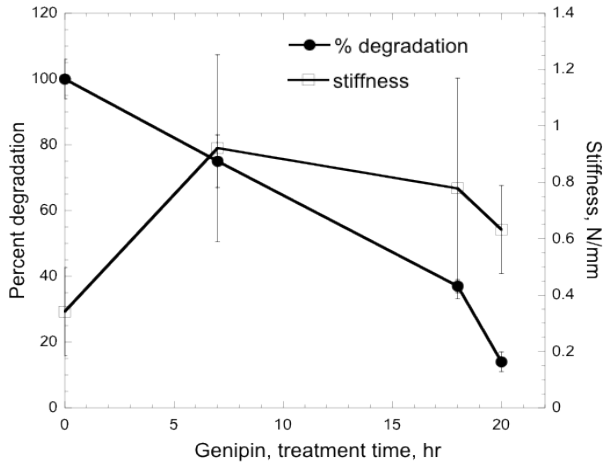


Figure 3. Effect of genipin crosslinking of amnion monolayers on membrane stiffness and degradation by bacterial collagenase. Amnion was treated with 0.1% genipin for varying times. * = $p < 0.01$ compared to value for control.

Genipin and carbodiimide appeared to produce crosslinked membranes with approximately equivalent beneficial properties. Since carbodiimide treatment drastically reduced the EGF level, it would be of great interest if genipin did not have the same effect. In a pilot study, treatment with 0.1% genipin for 20 h did not significantly reduce the level of EGF ($p > 0.05$). A full study was then carried out in which amnion was treated for varying times with 0.1% genipin in PBS. As shown in Table 4 and Figure 4, the decrease in EGF paralleled the decrease in percent degradation. Thus, genipin crosslinking of amnion did not show any advantage over crosslinking with carbodiimide with regard to loss of EGF; the desired result, namely, decreased enzymatic degradation while preserving EGF or other pro-healing proteins, was not produced under any of the treatment conditions evaluated.

Table 4 Effect of genipin crosslinking of amnion monolayers on EGF content and degradation by collagenase.

Treatment,	% enzymatic degradation ± SD	EGF, pg/mg amnion ± SD
Control, buffer only	100 ± 5	7.62 ± 1.16
Genipin, 5 h	64.1 ± 8.9	4.67 ± 0.74
Genipin, 10 h	24.7 ± 2.3	0.66 ± 0.16
Genipin, 15 h	10.6 ± 0.9	0.30 ± 0.16
Genipin, 20 h	9.23 ± 1.44	0.30 ± 0.07

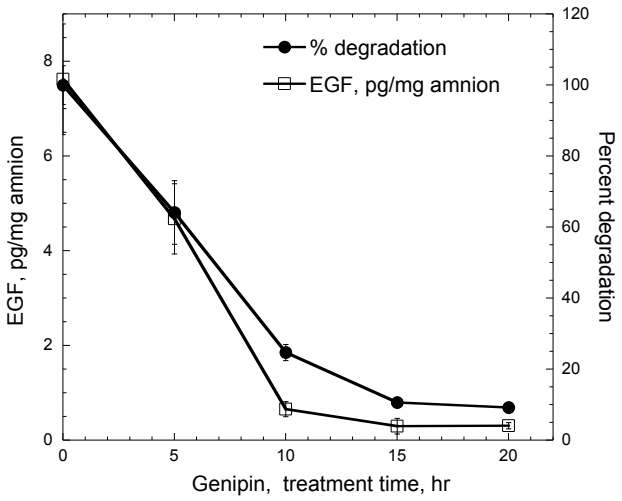


Figure 4 Effect of crosslinking monolayer amnion with genipin on EGF and enzymatic degradation. Amnion was incubated for varying times with 0.1% genipin. * indicates $p < 0.01$ compared to MES buffer control

Summary/ Conclusions

Both crosslinking treatments, carbodiimide and genipin, substantially reduced the level of EGF, a pro-healing factor in amnion, using conditions that produced an enzyme degradation resistant membrane that could be used for protecting cornea of ectropion eyes. This result suggests that we may not be able to achieve a goal of this project, namely, producing a protective membrane that retains the pro-healing factors in amnion. The results obtained with Rose Bengal (RB) photosensitized crosslinking may be promising since only partial loss of EGF was found but the results were confounded by residual RB in the membrane. Further studies in a no-cost extension period will be carried out to further evaluate the effect of RB photosensitized crosslinking on EGF in amnion.

Specific aim 1.e.ii. Identify the layering method that causes the least reduction in anti-inflammatory and healing factors in amnion.

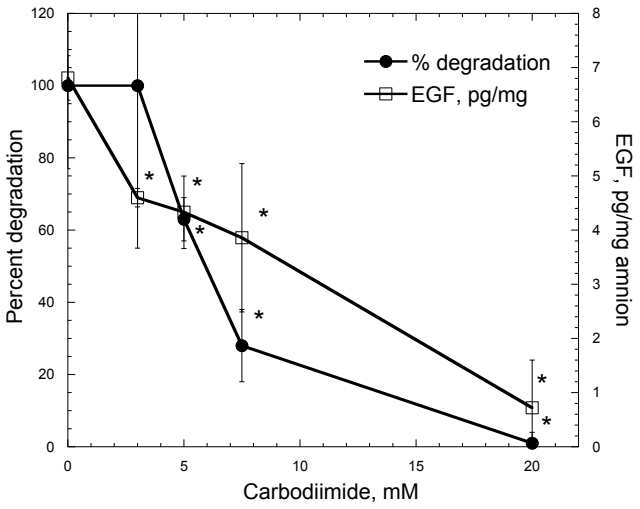
Tri-layer amniotic membranes offer the possibility that the pro-healing factors in the inner membrane might not be affected by the crosslinking and thus available to aid the cornea. In a pilot study, direct comparison of EGF remaining in monolayer and tri-layer amnion, both crosslinked by 7.5 mM carbodiimide, showed that >40% EGF remained in the tri-layer membrane whereas only 4% remained in the monolayer.

In a more detailed study, tri-layer membranes were prepared as described above under Specific Aim 1.b using concentrations of carbodiimide of 3, 5, 7.5 and 20 mM in MES buffer and a 1 h incubation time. The percent degradation by bacterial collagenase was measured and the soluble protein was extracted as described under Specific Aim 1.e.i above and used for measurement of EGF by ELISA. The results in Table 5 and Figure 5 show that, as expected based on the results reported in Year 2, these treatments protected the membrane from enzymatic degradation. The amount of EGF extracted also decreased with increasing carbodiimide treatment time. However, treating with concentration of 5 mM and higher carbodiimide produced membranes that were relatively higher levels of EGF in the tri-layer membranes compared to the monolayer membranes (see Figure 2). Possibly, the EGF in the middle layer of the tri-layer amnion was protected. Although this may be an interesting and useful observation, the stiffness of the tri-layer (as described earlier), does not allow it to be considered further as a protective membrane for ectropion eyes.

Table 5/ Figure 5. Effect on EGF of carbodiimide crosslinking tri-layer amnion membranes.

Cabodiimide treatment,	% enzymatic degradation ± SD	EGF, pg/mg amnion ± SD
0, MES buffer only	100 ± 23	6.81 ± 0.41
3 mM	100 ± 45	4.60 ± 0.17 *
5 mM	63 ± 6 *	4.33 ± 0.67 *
7.5 mM	28 ± 10 *	3.86 ± 1.37 *
20 mM	1 ± 3 *	0.72 ± 0.88 *

* indicates $p < 0.01$ compared to control value.



Summary/ Conclusions
Tri-layer amnion crosslinked by carbodiimide treatment showed better retention of EGF than similarly crosslinked monolayer membranes. However, the high stiffness of these enzyme-resistant crosslinked tri-layers precludes their use for cornea protection.

Specific aim 1.f.i. Evaluate the healing properties of crosslinked amnion on the rabbit ectropion model.

The rabbit ectropion model has been established by Dr. Johnson at USAISR as described in his annual report. We have prepared samples of monolayer amnion crosslinked with carbodiimide and untreated controls and measured their degradation by bacterial collagenase. Amniotic membranes that showed less than 15% degradation, relative to untreated controls, were sent (along with controls) to be tested on these rabbits to Dr. Johnson. We will continue to supply crosslinked amniotic membranes to Dr. Johnson (during the no-cost extension period) and make any adjustments indicated by the initial in vivo results.

Specific aim 1.f.ii. Evaluate multilayered amnion construct on the rabbit ectropion model.

The results obtained during Year 2 indicated that tri-layer, but not bi-layer, crosslinked amniotic membranes were more resistant to degradation by bacterial collagenase than monolayer crosslinked amnion receiving the same treatment. However, our results in Year 3 indicate that stable, enzyme-resistant tri-layer membranes, crosslinked with carbodiimide, are too stiff to conform to the contour of the cornea. This factor lead us to conclude that tri-layer amniotic membranes are not a feasible material to use to protect cornea of ectropion eyes. Consequently, they will not be evaluated in the rabbit ectropion model created by Dr. Johnson.

Specific aim 3.a. Construct amnion-covered hydrogel bandage.

In order to provide moisture and prevent tear evaporation from the cornea of ectropion eyes, we had proposed to seal a hydrogel contact lens within an amnion capsule by a crosslinking process and then bond this construct to the cornea using our light-activated crosslinking process. In Year 2 we fabricated amnion-encapsulated hydrogel lenses by photo-crosslinking technique Rose Bengal-stained amnion. We also carried out feasibility studies to test bonding of these constructs to cornea ex vivo. The results reported in Year 2 suggested that this approach was feasible. However, our previously reported results on RB photosensitization and additional recent studies indicate that this crosslinking technique decreased enzymatic degradation by a maximum of ~50%. Since degradation of the amnion during use of the encapsulated lens would shorten its useful lifetime, we tested an alternative method for encapsulating a hydrophilic contact lens in amnion.

Our alternative approach involved using carbodiimide to crosslink the two amnion layers of the capsule since this agent can protect the membrane against enzymatic degradation by >90%. This process bonded the two layers together around the circumference as well as making the amnions resistant to proteolytic degradation. The amnion-contact lens-amnion “sandwich” was placed between two copper fine mesh screens that were shaped to approximate the contour of the cornea anterior surface. The screens allowed the crosslinking reagents to diffuse into the

amnion, as shown previously when the tri-layer amnion constructs were made (Specific Aim 1.b).

The amnion discs were 13 mm diameter and the lens (1 DAY ACUVUE - TRUeye) was cut to 6 mm diameter. Approximately 3 mm at the circumference of the two amnion layers was held in tight contact to facilitate crosslinking between the two layers. In the "sandwich", the basement membrane surface of one amnion disc was in contact with the stromal surface of the other. The construct was placed in 10 mM carbodiimide/MES buffer and incubated with shaking for 1 h.

To test if this construct could be bonded to cornea ex vivo, we used the method reported in Year 2. Briefly, the material to be tested is stained with 0.1% Rose Bengal for 1 min and placed over the de-epithilialized cornea surface of an ex vivo rabbit eye containing a V-shaped incision. After irradiation with 532 nm light, the strength of the bond between the construct and the cornea was measured by infusing saline into anterior chamber and measuring the intraocular pressure (IOP) that causes leakage under the construct.

For the carbodiimide crosslinked encapsulated lenses, 200 J/cm^2 of 532 nm radiation was applied at an irradiance of 0.25 W/cm^2. The bonding strength was good: IOP = 54.7 ± 28.0 mm Hg (n = 6), after learning to adjust for good fit.

Summary/ Conclusions
Carbodiimide crosslinking can be used to produce amnion-covered hydrophilic contact lenses that can then be bonded to the cornea. This approach has the advantage, over of RB photosensitization, of producing a more enzyme-resistant covering.

Specific aim 3.b. Evaluate bonding amnion-encapsulated hydrating bandage on the rabbit.

We will be supplying Dr. Johnson with amnion-encapsulated contact lenses for evaluation on his rabbit model for ectropion during the no-cost extension period.

KEY RESEARCH ACCOMPLISHMENTS

- Identified the stiffness of crosslinked amnion membranes as a physical property critical for development of a cornea protective membrane that conforms to the shape of the cornea and quantitatively evaluated this property. Monolayer crosslinked amnion had sufficiently low stiffness to allow production of highly stable crosslinked membranes that can be used for cornea protection. The stiffness of tri-layer crosslinked amnion that was resistant to enzymatic degradation was too great for this application.

- Determined that two of the three crosslinking treatments that stabilize amnion against enzymatic degradation also substantially decrease the level of a soluble protein pro-healing protein, epidermal growth factor (EGF). Crosslinking with carbodiimide and genipin decreased EGF by >90%. Crosslinking with RB photosensitization requires further evaluation.

- Determined that tri-layer crosslinked amnion partially retained the EGF in the amnion but was unsuitable as a protective membrane because of its high stiffness.

- A protease-resistant amnion-covered hydrogel contact lens for providing hydration to the cornea was constructed using carbodiimide crosslinking and demonstrated to photobond to ex vivo cornea. This approach is an alternative to the Rose Bengal photosensitization previously demonstrated and provides the construct with a more enzyme-resistant membrane.

REPORTABLE OUTCOMES: None during this reporting period.

CONCLUSION:

In year 3, we have progressed toward our goal of developing materials to protect the eyes of patients with severe periorbital burns and scarring. These patients cannot blink normally or close their eyes (a condition called ectropion), leading to damage to the ocular surface and possibly the need for corneal transplant. Our approaches are to stabilize and modify amniotic membrane, a beneficial material for cornea surface treatment that cannot be used now because it rapidly degrades in these inflamed eyes, and to develop a hydrating contact lens that can be bonded to the cornea surface.

We have used protein crosslinking to produce amniotic membrane that is resistant to degradation by enzymes in the tears of inflamed eyes. In Years 1 and 2 we had identified two effective protein crosslinking techniques and in Year 3 we identified a third approach. We found that, in addition to providing a membrane that is stable against enzymatic degradation, the membrane must remain flexible enough to conform to the shape of the cornea. During this period we established a method for testing the stiffness of the crosslinked membrane. This testing allows evaluation of crosslinking treatment conditions without requiring ex vivo or in vivo testing. Crosslinked membranes that were resistant to enzymatic degradation were produced that had low stiffness and can be used for further in vivo evaluation. Tri-layer crosslinked amniotic membrane constructs, however, which showed excellent degradation resistance were too stiff to be used on corneas and will not be further considered. Another criteria for a successful cornea protecting membrane is that it retain the pro-healing factors present in amnion. We showed that two of the three protein crosslinking methods decreased substantially these factors in amnion; the third method is being further evaluated. We had previously developed an amnion-encapsulated contact lens designed to provide hydration to the cornea. We have now established an additional approach that provides a more enzyme-resistant crosslinked amnion covering that should extend the period that this lens provides hydration to the cornea and thus inhibit damage to the surface for patients who are unable to supply tears by blinking. These crosslinked amion-based materials and hydrdogel constructs are now ready to be tested on the rabbit model for ectropion developed by the partnering PI, Dr. Johnson.